

RDM

Recombinant DNA methods

Agenda

DAY 2

- cloning foreign DNA into a plasmid – show video
 - gel purification / isolation
 - ligation
 - explain our modifications to the protocol in the video
 - direction cloning
 - phosphatase treatment
 - inoculate ara+ and ara- overnight cultures for PCR analysis
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Cloning foreign DNA into a plasmid

- General strategy: cut foreign DNA and vector plasmid separately with the same restriction enzyme. Run these digests on a gel and cut out the foreign insert and the linearized vector (backbone). Use DNA ligase to join the compatible sticky ends of these two fragments, and then transform the resulting recombinant plasmid into bacteria. Grow the transformed bacteria overnight on an ampicillin plate. The colonies you see in the morning are descended from bacteria which gained ampicillin resistance by taking up intact plasmid during the transformation.
- Show video “Plasmid Cloning” from Molecular Cell Biology ed. 3.0, which explains this graphically.
- We are making two modifications to the protocol outlined in the video: directional cloning and phosphatase treatment.

Directional cloning

- In the cloning video, they used only a single restriction enzyme (EcoRI) to digest their vector and foreign DNA. We will use two. This allows us to insert the foreign DNA in a specific orientation. This is important, because the promoter is part of the vector, not the insert. The inserted gene has to go in with the 5' end of the coding sequence next to the promoter, in order for the protein to be expressed. What would happen if the insert went in backwards? (Might get transcription of the non-coding strand and expression of a nonsense protein. More likely, nothing at all will be expressed.)

Phosphatasing the vector

- In the example in the video, the use of only a single restriction enzyme left the vector backbone with two compatible sticky ends.
- It's much easier for two sticky ends to find each other if they're on the same molecule, compared to on different molecules, and so there's a high likelihood that the vector will simply re-circularize on itself.
- If a lot of vector molecules recircularize, we are likely to get many transformants with just vector DNA. We might have to sort through hundreds of colonies to find just one with the insert!

- DNA ligase requires a 5'-phosphate at the ligation junction. Using a phosphatase (such as CIP, Calf Intestinal Phosphatase) to remove phosphates from the vector backbone is a way of avoiding the recircularization problem.
- However, you have to get rid of the phosphatase before the ligation reaction, or else it will pull the phosphates off the insert DNA too, and nothing at all will get ligated! Gel-purification might be expected to separate the vector from the phosphatase enzyme, but in practice this does not work, and you always get some phosphatase in the band you cut out. (The enzyme likely runs as a broad—and of course invisible—smear on the gel)
- The way we get around this problem is by heat-killing the phosphatase. After the CIP reaction has been incubated at 37 deg for 10 min, we heat the reaction to 65 deg for 10 min. This harsh treatment irreversibly denatures the enzyme.
- Question: If one of the two components of a ligation reaction has been phosphatased, what does the product look like? Do both strands get ligated? (Answer: No. The product is left with a “nick”, but the bacterium can repair this once the plasmid gets inside it.)
- Note: Why couldn't we pick the insert to phosphatase instead? “Can't you just pick one or the other?”
Reasons why we can't:
 - The vector will still recircularize and give us amp-resistant transformants that lack the insert.
 - A self-circularized insert wouldn't be a problem in the first place: it wouldn't confer amp resistance, and so any bacterium that was transformed with such a circular DNA would die on the ampicillin plate.
- Note: It is OK if you incubate the insert at 65 deg too, as long as you don't put any phosphatase (CIP) into it.

Gel purification and isolation of insert and vector backbone

- The restriction digest of pUGFP yielded two fragments. How big are they? (2.6 kb and 1kb.) Which of them is the GFP insert (1 kb), and which is the backbone of plasmid pUC19 (2.6 kb)? Which do we need for our purposes? (GFP insert, 1kb) We will use an agarose gel to purify the insert.
- Although it may seem unnecessary, we will also purify the vector backbone. Why do we do this? Need to get rid of the restriction enzyme. Also, because we're cutting in two places, we need to get rid of the small excised fragment, which would interfere with our ligation reactions. The pET digestion produces bands at 4kb and 650bp. We will excise the 4kb band. Supercoiled undigested pET happens to migrate just below this 4kb band—have to be careful in choosing which band to cut out!
- We will use a low-melt agarose gel to perform these gel purifications. Why? Allows us to simply cut out the DNA band and melt it, yielding a warm solution of agarose and DNA. We can then add ligase and ligase buffer and let the agarose re-solidify. The ligase works even inside the agarose! If we used regular agarose, we wouldn't be able to easily melt the gel slice.
- Low-melt gels are extremely fragile! This point cannot be overemphasized. Don't pull the casting tray out of the gel tank until *after* you've removed the comb out. Removing the comb while the gel is immobilized like that makes breakage less likely. Also, never pour TAE buffer directly onto a low-melt gel—it will break! Pour the TAE into the reservoirs at either end of the tank, or simply lower the cooled gel into a pre-filled tank. After you've run your gel, it will have heated up. This makes it even more fragile. Wait a few minutes after shutting off the voltage, for the gel to cool a little, and then pick up the gel still in its tray, and put the whole thing on a piece of saran wrap to bring to the gel-cutting station.
- The gel-cutting stations have a long-wave UV light. Why use long-wave UV? It has less energy and causes less damage to the DNA. We want to limit the amount of UV energy that the DNA absorbs. Limiting the exposure time helps too.
- Remember to wear gloves and goggles when working around EtBr and UV light!

- Make sure to use a fresh razor blade for each band you cut out. It is important to avoid cross-contamination of bands. Be especially careful not to mix the undigested vector with the digested vector, as this will yield numerous false-positive transformants.

Ligation

- We will set up four ligations. Only one of them has both components in it (vector and insert.) The other three have just one component in them, and such they are not really ligation reactions at all but controls on various aspects of the experimental protocol.
- One reaction will have just the digested vector. This is a way of testing for how complete the digestion and phosphatase reactions were. What should this “ligation” yield when used to transform bacteria? (Answer: nothing, unless the vector was incompletely digested, or it recircularized due to incomplete phosphatasing.)
- One reaction will have just the insert DNA. This is a way of testing how careful you were to avoid cross-contamination of the gel slices. What should this “ligation” yield when used to transform bacteria? (Answer: nothing, unless you isolated some vector with the insert slice.)
- The last reaction will have just undigested vector in it. This is a way of testing whether the presence of agarose prevents the cells from taking up DNA. What should this “ligation” yield when used to transform bacteria? (Answer: Lots of transformants, except if the agarose inhibits transformation.)
- Make sure to add ligation buffer to each ligation reaction.
- Make sure the agarose is completely melted before you add ligase! Otherwise the DNA will be trapped in the agarose and the ligase won't be able to get to it.
- As always when working with enzymes, keep the enzyme tubes on ice when not pipeting from them, hold the tubes from the top, and just touch the tip of the pipet tip to the surface of the liquid, in order to avoid wasting enzyme.
- After adding enzyme to the ligation reaction, put the tubes on ice and carry them to the thermocyclers (PCR machines). Reactions will be incubated in the PCR machines at 15 deg overnight. Why such a cool temperature? Answer: helps the sticky ends anneal.

Inoculation of ara+ and ara- strains for PCR analysis

- We will grow up two cultures of bacteria from the GEN module: one of them is the “wild-type” ara+ strain with an intact arabinose pathway, and the other is one of your ara- mutants, with either constitutive or inducible expression of LacZ from a transposon disruptively inserted into the AraDABC operon.
- On Day 3, we will isolate genomic DNA from each of these two strains using a boiling lysis method. Then we'll use specific pairs of PCR primers to selectively amplify three regions of the Ara operon, corresponding to the A, B, and C genes. The results of this analysis will tell us which of these three genes had the LacZ-carrying transposon inserted into it.
- We'll talk more about PCR and the analysis of the ara mutants in the Day 3 recitation.
- For now, just remember to use sterile technique when inoculating these cultures. Use a sterile stick to lift a single colony off the plate, and drop the whole stick into a tube with 2 mL of LB. (Do we want to add ampicillin to this LB? Why or why not?) Then cap and label the tube and leave it in the rack in the cold room. Do not put it in the warm room! Two days is too long for the bacteria to go without fresh media. We want them to be fresh when we isolate their DNA. Just leave the tube in the cold room, and the teaching staff will move it to the warm room before your next lab day.